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In re: Walsh et al.
Appl. No. 09/689,430
Filed October 12, 2000

APPENDIX A

3. Coarest of FH in day 1 supernants of ~~HBV~~pdbr2 infected 3T3 & HepG2 cells

TABLE OF ABSORBANCE VALUES

FILE: FILE 1 TITLE: FILE 1 REPORT DATE AT 05:40 PM

	1	2	3	4	5	6	7	8	9	10	11	12
delHBV → A	0.066	0.020	-0.006	-0.060	-0.080	-0.099	-0.105	-0.108	-0.093	-0.103	-0.111	-0#110
	20	14.3	9.1	4.8	1.2	Blank	3T3/F8	3T3	DMEM	HepG2 F8	HepG2	MEM
duplicate B	1.201	0.944	0.705	0.495	0.296	0.220	0.227	0.232	0.221	0.218	0#234	0#426
C	1.025	0.916	0.660	0.495	0.305	0.245	0.073	0.296	0.247	0.224	0#213	0#224
Nocling → D	-0.123	-0.120	-0.124	-0.121	-0.119	-0.119	-0.123	-0.124	-0.121	-0.122	-0.122	-0#124

4. Re-set Ultra-spin of rBA/F3 viruses from 40 plates.

tube ④ ⑤ ⑥ ⑦ ⑧ → 2 × tubes

others → 2 × tubes.

4°C 15°C × 6 h.

⑥ feed 3T3 & HepG2 /DG2 at 9:00 pm

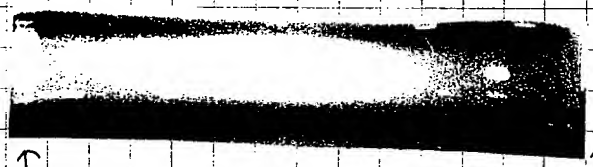
⑤ PCR of Enhancer I of HBV. 2×

delta	26 ml	template	(0 ml (HBV/pUC19)
(0 × Buffer	5 ml	PFu (Stratagene)	1 ml (2.5u)
dNTPS	4 ml		10 ml
5'-P	2 ml (40 pmol)		
3'-P	2 ml (40 pmol)	mineral oil	40 ml

95°C 2 min
95°C 2 min
50°C 1 min
72°C 3 min
} 8 cycles.

95°C 2 min
57°C 1 min
72°C 3 min
} 20 cycles. → 72°C x 10 min
↓
4°C

loading PCR product → 2% agarose gel.



↑ 50 bp DNA ladder

100 bp from 50 ml system

Coatest of Day 2. 3. 4.

TABLE OF ABSORBANCE VALUES

FILE: TITLE: FILE 1
FACTOR: 1.000

	1	2	3	4	5	6
A	-0.002	-0.005	-0.003	0.001	-0.001	0.000
	20	14.3	9.1	4.3	1.4	0
double standard B	0.748	0.645	0.587	0.427	0.308	0.311
C	0.754	0.559	0.622	0.456	0.336	0.322
D	0.316	0.252	0.327	0.297	0.282	0.325
E	0.369	0.230	0.352	0.368	0.339	0.379
F	0.346	0.268	0.350	0.340	0.331	0.358
G	0.337	0.262	0.334	0.344	0.339	0.356

No f g ?

Day 5. 6 Sample

TABLE OF ABSURBANCE VALUES

FILE: TITLE: FILE 1 DATE: AT

FACTOR: 1.000

	1	2	3	4	5	6	7	8	9	10
	0	1.4	4.3	9.1	14.3	20?				
double standard A	0.265	0.355	0.470	0.648	0.847	0.993	0.001	0.004	0.001	-0.003
B	0.285	0.351	0.487	0.702	0.935	1.201	0.016	0.002	0.015	0.002
C	0.296	0.292	0.283	0.288	0.285	0.295	0.311	0.280	0.344	0.298
D	0.300	0.310	0.337	0.319	0.342	0.325	0.326	0.302	0.256	0.314

No f g

Sequency of TLC promotor is ok this time.

(1) Partial Digestion of pLZ₁-N₃ with BglII

from the previous experience BglII can not cut pLZ₂ well. so use relatively more BglII for partial digestion

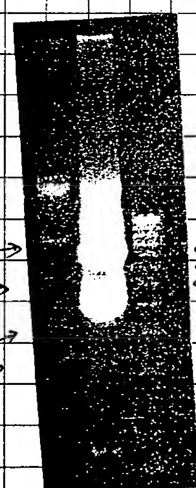
ddH₂O	8 ul	
10x React buffer 3	4 ul	
pLZ₂-N₃	25 ul	(8 ug)
BglII	3 ul	
	40 ul	37°C 1 hr

load to 0.7% agarose gel

cut the band between 7-8 kb.

purify through Qiagen gel kit

elute DNA in 100 10mM Tris.Cl (pH8.0)



(2) purify EphL pcr products (100ul) by QiaEX II kit

Elute DNA in 40ul 10mM Tris/ (pH8.0)

③ EnhI / BamHI + HindIII

ddH2O	
10x New BamHI Buffer + BSA	5.5
EnhI	40 ul
HindIII	2 ul
BamHI	2 ul
	50 ul

37°C overnight

① add 1 ul HindIII
1 ul BamHI
1.1 ul Buffer + BSA
6.9 ul ddH2O

37°C 4 h

② loading to 2% agarose gel.

③ purify the Bands by QIAEX II. elute DNA in 20 ul 10 mM Tris-Cl (pH 8.0)

④ Ligation

① [illegible] 6 µl

[illegible] 2 µl

② [illegible] 6 µl

[illegible] 2 µl

③ [illegible] 6 µl

[illegible] 1 µl

10× Ligase buffer 1 µl

Ligase 1 µl

(Clontech)

16°C 18 h (overnight)

Transformation: 100 µl bacteria to each plate

① 7 colonies

② ≥200

③ ≥ 200 → pick 20 colonies from ③

→ 3 ml LB/Amp 32°C overnight

① Extract DNA ② Digest with SmaI

① 11kb DNA ladder

②–⑳ : 1st~20 th colony (two tiny) were cost

㉑ XhoI+HindIII

colony 1. 2. 7. 9. 10. 11. 12. 13. 14. 16. 18 have two ITRs.

cut 11 colonies with both ITRs with BglII + XhoI

ddH2O	2 μl
10×React 3	4
DNA	30
BglII	2
XhoI	2
	40 μl

37°C 2 h

① EcoRI right orientation between 5'ITR & TIC → 255/80

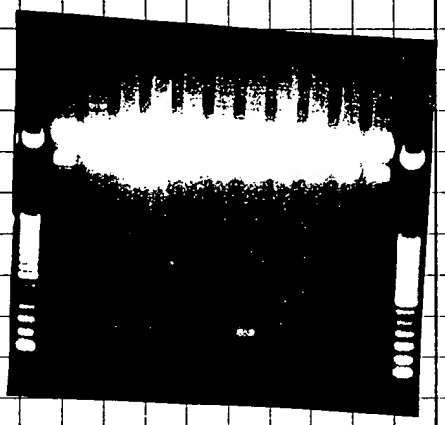
② ~~---~~ & p(A)n → 122/200

③ reverse orientation at 5' → 122/80

④ reverse orientation at 3' → 122/80

⑤

I can not see
250bp Band in any lane.



pick another 20 colonies from plate 2
→ 3ml LB/Amp 37°C overnight

Digest DNA, cut with BglII + XhoI
No 450bp band.

increase amount of EcoRI, set ligation again

DUB2/[illegible] + CIP	4 ul
EcoRI	4 ul
T4 ligase	1 ul
10x Buffer	1 ul

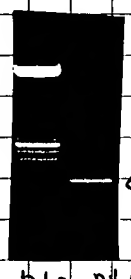
16°C 20 h

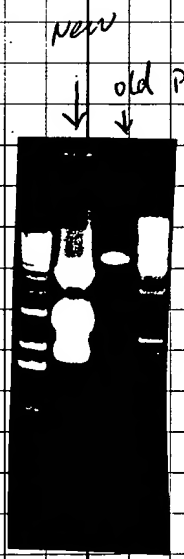
Transformati

The colonies on control (only pCZ2/MCS)
= pCZ2/MCS + EcoRI
① forgot to do dephosphorylati

Reset pCZ2/MCS Partial Digest
ddH2O 8 μL
10× React 3 4 μL
pCZ2-N3 25 μL
BglII 3 μL (Gibco)
40 μL 37°C 1 h
load to 0.7% agarose gel
Complete Digest!

Reduce the Enzyme to 1 μL
cut 7.5 kbp Bands (vector)
1400 bp Bands (inhomer)
elute vector in 40 μL
EcoRI in 20 μL
room temperature (RT)





DEP vector.

DNA 39 ml
dd H2O 2 ml
10x Ligation buffer 5 ml
T4 DNA ligase 4 ml
(NEB)

50 ml 37°C 2 h

purify through QiaEX II kit
elute DNA in 20 ml 10mM Tris pH

2. ligation.

① Vector 4 ml
dd H2O 6 ml

② Vector 4 ml
insert 4 ml

③ Vector 6 ml
insert 2 ml

④ Vector 6 ml
insert 1 ml
dd H2O 1 ml

+ 1 ml ligase
1 ml 10x buffer.
16°C overnight

Transformation

① There are similar number of colonies (7-9/plate) on ~~control~~ control and vector + insert plate

I do not know the reasons.

① poor-digested ends of the PCR-origin enhancer I?

② The fragment of assumed DCE_2 are not the real one or the ?

Talk with Chris. I will start at very origin.

② Digest EcoRI PCR product with BamHI,

10 ul from 20 ul of 4/18 (P 3) PCR product

ddH2O 6.8 ul
10 ul DNA
10 x NEBuffer BamHI, 2.2 ul + BSA
BamHI (NEB) 1 ul
20 ul

37°C overnight 15 h

Add ddH2O 7.9 ul
10 x NEBuffer + BSA [illegible] ul
BamHI 1 ul

/ 10 ul → 37°C 8 h

1. purify EcoRI/BamHI, by QIAEX II kit, elute DNA in 20 μl Tris-Cl (10mM, pH 8.0)

2. Ligation

EcoRI/BamHI	4 μl
T4 DNA ligase (Promega)	4 μl
(3/20 P22)	
T4 ligase (Promega)	1 μl
(Promega)	
10× Ligase Buffer	1 μl
	10 μl, 16°C, 4 h

3. PCR:

dH2O	26 μl
dNTPs	4 μl
10× Buffer	5 μl
A-P	2 μl (3' primer of P(A)n)
S-P	2 μl (5' primer of EcoRI)
template	10 μl (ligation solution)
PFU	1 μl
	50 μl
mineral oil	50 μl

95°C, 2 min.

95°C 2 min
50°C 1 min | 6 cycles
72°C 5 min

95°C 2 min
57°C 1 min | 24 cycles
72°C 5 min

72°C X15 min

4°C

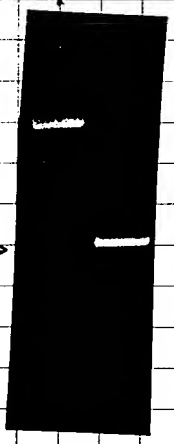
load the 2% agarose gel

cut the band at 350bp

purify by QIAEX II kit

elute in 40 μl X2 10mM Tris-Cl

20 μl 50bp DNA ladder (2 μg)



4. Test cut by XhoI

dd H2O	0
10×NEBuffer 2+BSA	4.4 μl
DNA	32 μl
XhoI	3 μl
	30 μl

37°C 2 h

5. by - BglII.

ddH2O	0
DNA	41 uL
10×React 3	5 uL
BglII	4 uL
	50 uL

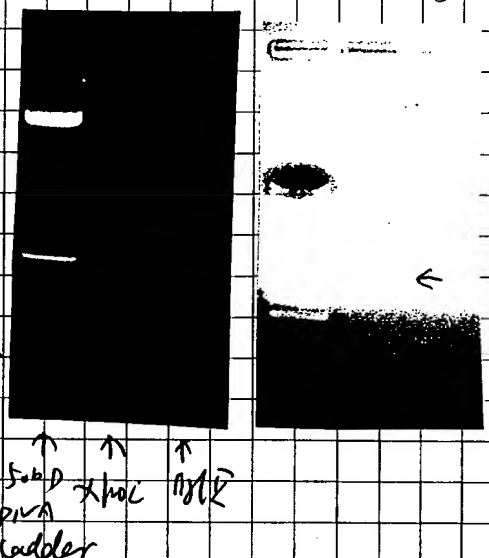
37°C overnight

① Add { ddH2O 8 uL; 10×React 3 1 uL; BglII 1 uL } ⇒ Ligase / BglII

37°C 10 h

② load to 2% agarose gel

≈250 bp Band and 80 bp Band can be seen in XhoI lane



③ cut the BglII Band. purify through Qiagen kit. elute DNA in 20 uL 10 mM Tris-Cl

④ Ligation

② PTR/NdeI insert 6 µl (Page 66)

Entry vector PA 2 µl → pDEST8

① PTR/NdeI insert 4 µl

ddH2O 4 µl

1 µl T4 ligase + 1 µl 10× Buffer

16°C overnight

Transformation

① 14 colonies on plate 2

2 plate 1

Bad ligase?

pick 12 from plate to 3 ml LB/Amp

37°C rocker-drum overnight

③. Ligation

4 ul EnhI/BamHI (P.103)

4 ul (IL+MCS+pCA) (Day 1 from colony 4 & 7 of p(Z)3)

⊕ T4 Ligase 1 ul (NEB)

10x buffer 1 ul

⊕ in notebook for making pPLZ4.

16°C overnight

④ PCR

ddH2O	26 ul	
dNTPs	4 ul	
A-P	2 ul	(3' primer of P(A)n)
S-P	2 ul	(5' primer of EnhI)
template	10 ul	(ligate system)
Pfu	1 ul	
	50 ul	

95°C 2 min

95°C 2 min
50°C 1 min
72°C 5 min
/ 6 cycles

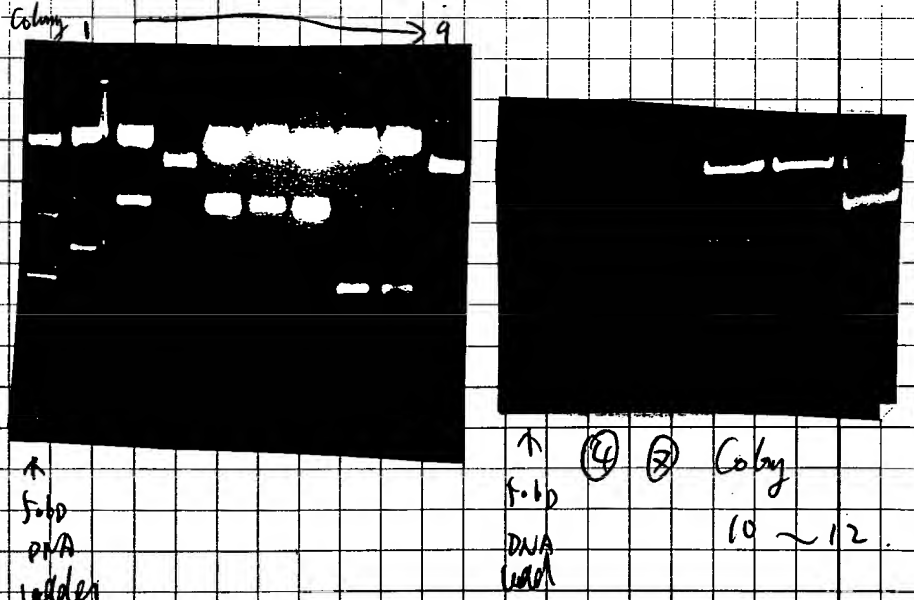
95° ~ min
57 1 min
72°C 5 min
/ 24 cycle → 72°C X 15 min
↓
4°C

② Extraction of plasmids pdtZ & by Promega wizard mini-prep kit, elute DNA in 50 ul dd H2O.

③ Digestion with BglII

dd H2O	3 ul
10 x Buffer 3	4 ul
DNA	30 ul
BglII	3 ul
	40 ul. 37°C 2 h.

load to 2% agarose gel



Colony 7, 8 seem right insertion

cut PCR product of EcoRI + TK + MCS + pCAT (500 bp) elute DNA in 60 ul Tris-Cl (10 mM, pH 8.0).

(4) make more plasmids of colony 2 & 8.

(1). Enh2 + TK + MCS + p(A) ⟶ ETMP. / BssHII

deH₂O	3 ul
10x NewBuffer	
BSSHII	4 ul
DNA	30 ul
BSSHII	3 ul
	40 ul. 50°C 2 h

BglII.

deH₂O	11
10x React 3	5
DNA	30
BglII	4
	50 ul

37°C overnight.

(2). DLZ5 / XhoI

deH₂O	0
10x NewBuffer 3 + BSA	5.5 ul
DNA	41 ul
XhoI	4 ul
	50 ul. 37°C 2 h

(3) DLZ5 / SmaI

deH₂O	10 ul
10x React 3	3 ul.
DNA	15 ul.
SmaI	2 ul
	30 ul RT 2 h.

load to 2% agarose gel

M: 50bp DNA Ladder

1 C7/SmaI

2 C8/SmaI

3 C7/XbaI

4 C8/XbaI

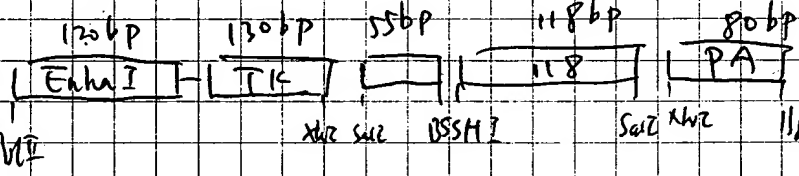
5 ETMP-4/BSSHII

6 ETMP-7/BSSHII

①: ψ at one ITR was deleted.

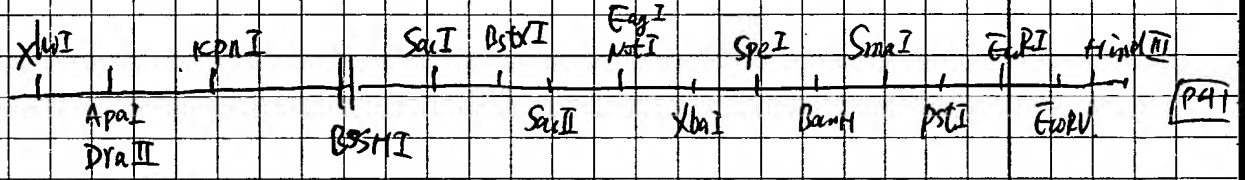
② seems no repeated insert of DC2-5 →

③ ETMP-4: /BSSHII ≒ 300 + 200

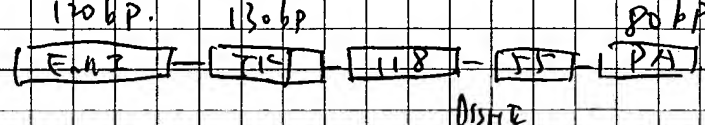


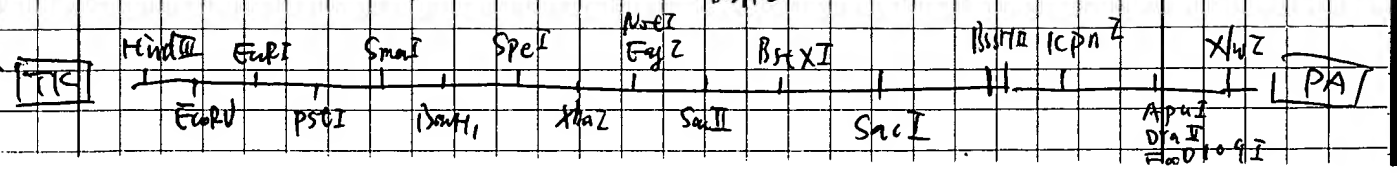
Results are comparable to results of SmaI Digestion of TIC+ITR+tPA.

Rearrangement of MCS.



ETMP-7 ≒ 320 + 140





① Add BglII to ETMP/BglII system.

{ ddH₂O 16 ul
{ 10x React 3 2 ul
{ BglII 2 ul
20 ul   37°C. 6 h

② Extract DLZ5/XhoI from gel.
elute DNA in 50 × 2 10 mM Tris-Cl.

③ DLZ5/XhoI / BglII

DLZ5 Colony 7 & 8
both OK

DNA 41 ul
10x React 3 5 ul
BglII 4 ul
50 ul   37°C 2 h.

④ DLZ5/XhoI / CIP.

DNA 60 ul
10x NovaBuffer 3 8 ul
CIP 6 ul
ddH₂O 6 ul
80 ul   37°C 2 h.

load to 2% agarose gel.
0.7%.

DLZ5 C7 XhoI/BglII
C8
500bp DNA ladder

ETMP-1
ETMP-4

{ 4 ul 100 mM EDTA
{ 75°C × 10 min
{ purify by QiaEX
elute DNA in
20 ul TE
use colony 7 for next
[illegible]

(5). pTR EGFP from Rebecca.
swap EGFP replaced of GFP in pTR-UF5.
Digest with XbaI + BclI (BM) to get

EGFP + PA + TK + Neo + cassette = 2265bp.
vector = 4010.

ddH2O	17 uL
10x Sure cut/H	4 uL
DNA	15 uL
BclI	2 uL
XbaI	2 uL
	40 uL

37°C 2 h.

← pTR/EGFP

BclI + XbaI

↑ 1kb DNA ladder

load ~~[illegible]~~ to 0.7% agarose gel.

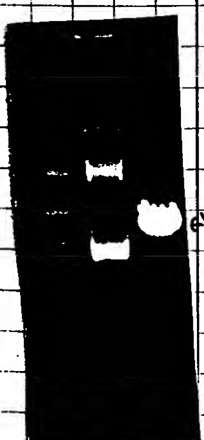
Not cut by one enzyme:
BclI? or XbaI?

figure out which is bad.

ddH2O	23 uL
pOLZ2	2 uL (1 ug)
10x Buffer H/M	3 uL
BclI / XbaI	2 uL
	30 uL

37°C >2 h.

Load to 0.7% agarose gel.
BclI is bad



XbaI

↑ BclI

← cut vector band
purify by Qiagen
gel kit. elute
DNA in 100 uL Tris.

↑ pTR-EGFP ↑ pOLZ2-N3 / BclI + XbaI / XbaI,

⑧. CIP of PTR/BglII.

dd H2O
DNA 98 ul.
10 x buffer 3 12 ul.
CIP (NEB) 10 ul

120 ul . 37°C . 1 h.

6 ul 100 mM EDTA 75°C x 10 min.

Desalt and concentrate by QiaEx II kit.

Elute DNA in 20 ul 10 mM Tris-Cl (pH 8.0)

⑨. Ligation

A . pCZf-C7/XhoI+CIP 4 ul. AA and F8/XhoI
dd H2O 4 ul.

B . pCZf-C7/XhoI+CIP 4 ul
FVIII/XhoI . 4 ul.

C . PTR/BglII+CIP 3 ul.
dd H2O 5 ul.

D . PTR/BglII+CIP 3 ul
EMP/4 5 ul

E. ETMP/7 just.

1ml of ligase & 10× buffer.

16°C overnight

Transformation. 100 ml cells/dish

only 13 colonies on plate B

4 on D

2 on E

No colony in amp ctrl → Bad ligate? Control of transformation is ok.

pick colonies → 3 ml LB/amp

37°C overnight

Repeat plating 200 ml cells on every dish

No improvement seen of colony number

Extract the plasmid by Promega Wizard miniprep kit elate DNA in 50 ml dd H2O

Digestion.

① EnhI + TIC + A8 + P(A) / XhoI.

ddH2o	14.7 ml
10 x NewBuffer 3	3.3
BSA	
DNA	10 ul
XhoI	2 ul
	30 ul. 37°C 2 h. (Overnight)

load to 0.7% agarose gel

① 1kb DNA ladder

②-⑬ Colony B1-12 / XhoI.

⑭ dfI / SmaI

② PTR + ~~EnhI~~ ETMP / BglII

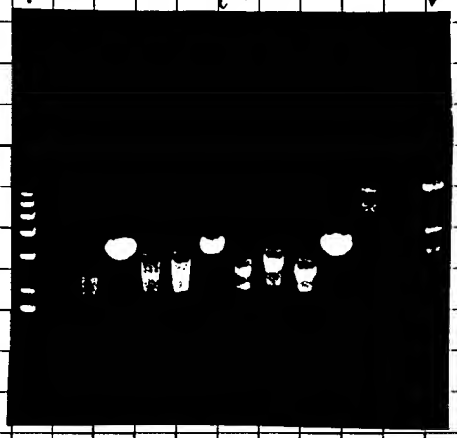
ddH2o	3 ul
10x Buffer 3	25 ul
DNA	
BglII	2 ul
	30 ul
	30°C overnight

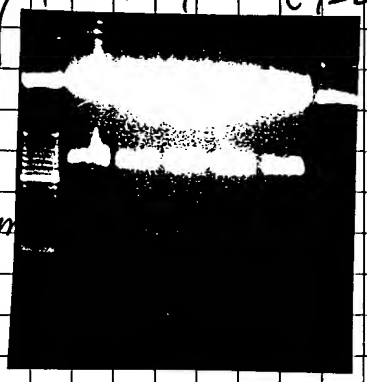
load to 2% agarose gel

① 50 bp DNA ladder.

②-⑤ Colony D1-4 (ETMP 4)

⑥-⑦ Colony E1-2 (ETMP 2)





① No insert of F8 to vector

② ETMP4 has 4 insert

③ 7 1 insert

Load 5 ul of F8/XhoI & D(2,5)/C2
to 0.7% agarose gel

the failure of ligation perhas is
due to incorrect ratio between
insert / vector

this time I will use ① { F8 = 6.0 uL
Vector = 2 uL

② { F8 = 5 uL
Vector = 3 uL

for ligation

1. Ran check of cold ligat system on 0.7% agarose gel.

① vector / ctp.

② vector.

③ 4 ul { vector / insert.

④ 3 ul vector
5 ul insert.

⑤ 2 ul vector.
6 ul insert

It seemed little ligat occurre even if there were some.
A little bit of bands can be seen on lane 3 – 5
Do transformat see what will happen

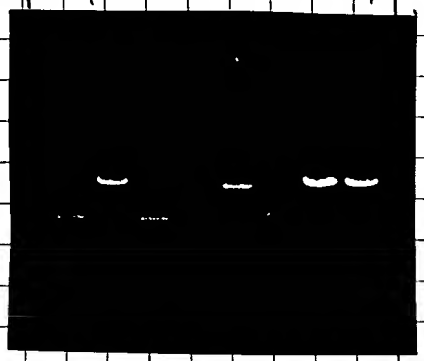
2. Pick another 4 colonies on old ligat
→ 3 ml LB/Amp

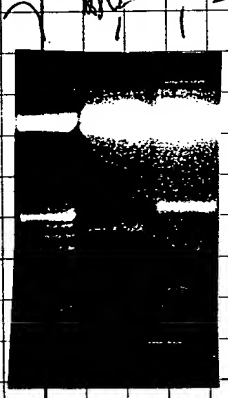
1. No colony on plate

2. mini prep of plasmids by promega kit
   elute DNA in 50 ul dd H2O

① cut with xho I.
   colony 9 has insert

② cut with Bgl II + Xho I.
   Bgl II + Xba I





a: Xho I + Bgl II Bands show right vector with right insert

b: Xba I + Bgl II show backward insert

I have two options for next step

① get more colony 9 cut with Xho I then re-ligated I should be able to get right insert + vector with right orientation

② Reprepare the vector/cap + F & and re-do ligation

first I will do ②

3. prepare the new batch of vector and FVIII.

① FVIII

ddH2O	22.6 ml
10× NEBuffer 2	4.4 ml
BSA	
pBCL2-C6	10 ml (2 ug)
XhoI	3 ml.
	40 ml 37°C overnight 2 h

② Vector

ddH2O	14.2 ml
10× NEBuffer 2	3.3
BSA	
DCL5-C8	20 ml
XhoI	2 ml.
	40 ml 37°C 2 h

run the 0.7% agarose gel. get the bands with clean gel kit

③ Dephosphorylation of vector

ddH2O	①
10× NEBuffer 3	5 ml.
DNA	40 ml.
CIP	4 ml
	50 ml. 37°C 1 h.

Add 2.5 ul 100 mM EDTA → 5 mM

75°C 10 min

purify through QiaEx II kit, elute DNA in 20 ul room tricl

4. Ligation

① D(7.5/Xho I+CIP) 4 ul
ddH2O 4 ul

② F8 4 ul

③ D - 4
F - 4.

④ D - 2
F - 6.

⑤ D - 1
F - 7

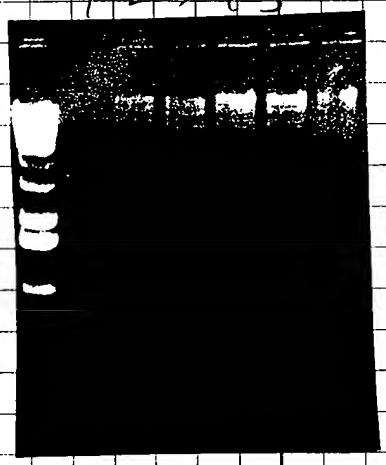
⑥ D - 0.5
F - 7.5

16°C overnight

① run the ligate gel.

cut from rest of each system.

Compared to May 18's ligate picture.



① the insert (F8) were ligated.

② the vector no obvious change.

Do the Dephosphorylate damage the DNA?

② Do transformation.

3. 4. 5. 6.

the number of colonies are

3 > 4 > 5 > 6.

pick 10 colonies from 3 & 5.

to 3 ml LB/Amp → 37°C overnight.

① Digest pIR-EGFP/XbaI with BclI (Brand new from NEB)

dd H2O	0
10x NEBuffer 3	13 ul
DNA (P113)	112 ul
BclI	5 ul
	130 ul

50°C 2 h overnight

② pIR-EGFP/XbaI + BamHI

dd H2O	10.5 ul
DNA	30 ul
10x BamHI buffer + BSA	5
BamHI	2 ul
XbaI	2 ul
	50 ul

37°C 2 h

load to 0.8% agarose gel.

pIR-EGFP vector can not cut by BclI.

purify DNA from Gel by Qiagen gel kit. elute DNA in 100 ul 10mM Tris-Cl (pH 8.5)

③ Re-cut the pTA-eGFP by XbaI or BclI to see at last what happened and will happen

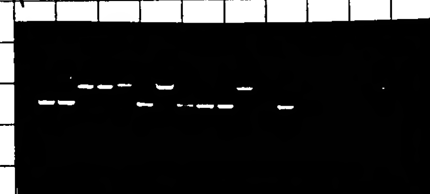
ddH2O	1 ul	0.4 ul	
10 X NEBuffer3	6 ul	6.6 ul	10 X NEBuffer 2 + BSA
DNA	48 ul	48 ul	
BclI	5 ul	5 ul	XbaI
	60 ul	60 ul	
	50 °C	37 °C	
	overnight	overnight	

④ Extract plasmid pCZ + F8 by Promega mini prep kit elute DNA in 50 ul ddH2O

cut with XhoI

ddH2O	14.7 ul
10 X NEBuffer2 + BSA	3.3
DNA	10 ul
XhoI	2 ul
	30 ul

37 °C 2h



Load to 0.7% agarose gel

3-5, 5-2, 5-4, 5-9 have insert of F8.

① further cut by BglII + XbaI
BamHI + XbaI.

BglII + XbaI — BamHI, + XbaI

	BglII + XbaI	BamHI + XbaI	
ddH2O	12 ul		
10X ~~[illegible]~~ buffer 3 + BSA	4.4 ul	4.4	10X NewBuffer Buffer + BSA
DNA	20 ul		
BglII	1.8 ul	1.8 ul	BamHI,
XbaI	1.8 ul	1.8	XbaI
	40 ul.		

37°C 2 h.

Load to 2% agarose gel.

① 50bp DNA ladder.
② 3-5 / BglII + XbaI
③ 5-2 / ——
④ 5-4 / ——
⑤ 5-9 / ——
⑥ 3-5 / BamHI + XbaI
⑦ 5-2 /
⑧ 5-4 /
⑨ 5-9 / ——

From

I picked 5 colonies from ligate DC2 s/XhoI + F8.

Got 7 colonies with F8 insert, 5 backbones (vector) are without EnhancerI (possible contaminats from F8/Xho with undigested DC2) with 2 with EnhancerI, only one has right orientation. It is today's colony 13.

50bp DNA ladder ↑ C13/XhoI + BglII = 250 + 80 ↑ C13 XbaI + BglII = 90 + ~

Culture 200 ml bacteria with Colony 13

(I failed to get DNA of DL26-C13 until so the following were done on)

- Extract plasmid of Colony 13 by Qiagen maxi kit. elute DNA in ml TE pH8.0

Digest with.

SmaI XhoI BglII

AgeI.

0.7% agarose gel:
1. XhoI (2) 4435+3500
2. BglII (3) 1945+2817+3173
3. SmaI (1) linear 7815 / 3013+4900
4. AgeI (4) 1235+6700.

2% agarose gel:
5. XhoI + BglII 250+80
6. XbaI + BglII. 190
7. XbaI + BamH1 190

50bp DNA ladder
XhoI + BglII
XbaI + BamH1

XbaI + BglII

- cut PTR/UFS & PTR-EGFP with BclI

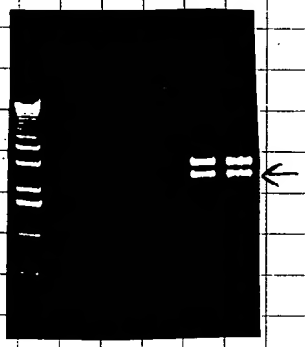
No BclI site along PTR-EGFP or UFS

I will cut PTR-EGFP with XbaI + BglII to get the EGFP + Neo cassette.

EGFP/BclI
UFS/BclI

PTREGFP/XbaI	98 μl	(P123)
10 x React 3	12 μl	
BglII	10 μl	
	120 μl	37°C 2 h

Load to 0.7% agarose gel



PTR-EGFP/XbaI + BglII

⇒ 4982 (EGFP+neo)

+ 3123 (vector)

(615 CMV promoter)

Cut the bands. purify through Qiagen gel kit

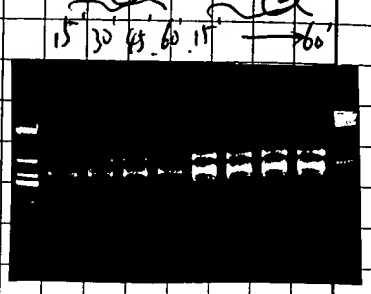
elute DNA in 30 μl 10mM Tris-Cl. (pH 8.0

1. Get pDL26-CB3 DNA digest (see last Page)
2. Partial Digest with BglII.

①	40 μl	DNA
	5 μl	10x NEBuffer 3
	3 μl	BglI
	2 μl	BglII
	50 μl	37°C 15' 30' 45' 60'

Add 10 uL 0.1M EDTA at every time point.

Load to 0.2% agarose gel.

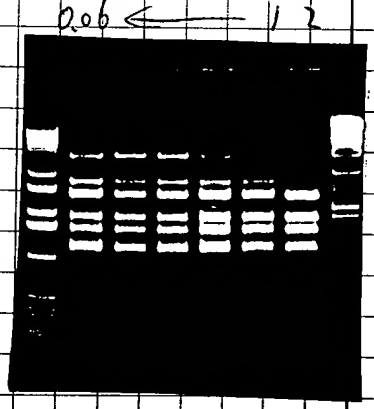


① BglII digest after 1set.

② Co-digest with 1st A at simultaneously.

EDTA could not stop restrict digestion or too much enzyme or too long incubate.

3. 40 uL DNA
5 uL 10x buffer 3
3 uL NheI
① 2 uL BglII
② 1 uL BglII (in 2 uL)
③ 0.5
④ 0.25
⑤ 0.125
⑥ 0.06



37°C 30'

Load to 0.2% agarose gel.

Cut the bands between 4.3kb & 5kb marker.

Purify DNA by QiaEX II kit. elute DNA in 20 uL 10mM Tris.Cl.

4. Ligate

F8: ① F8 4 µl + ddH2O 4 µl. No colony

② PTR/MI + CZP 4 µl + ddH2O 4 µl —

③ F8 4 µl
Vector 4 µl. 25 colony

④ F8 6 µl
Vector 2 µl. 7 colony

⑤ F8 7 µl
Vector 1 µl. 15 colony

EGFP. ~~① Vector~~
~~② EGFP~~

② EGFP 4 µl
Vector 4 µl 200–300?

① Vector 4 µl
EGFP 4 µl. >300

③ Vector 2 µl
EGFP 6 µl. >300

④ Vector 1 µl
EGFP 7 µl. >300

16°C overnight

① Transformation 200 μl Bacteria / 0.5 h

② prepare 2 liter culture of XX6 in the course, at least 1/3 were lost by contaminations. However, I still got 5 mg total (see last page) for transformation

pick 24 colonies from F8 plates

pick 6 colonies from EGFP① plate

→ 3 ml LB/Kan/Amp

(14 from F8, 6 from eGFP)

Extract plasmids by Promega miniprep kit

Elute DNA in 50 μl dd H2O

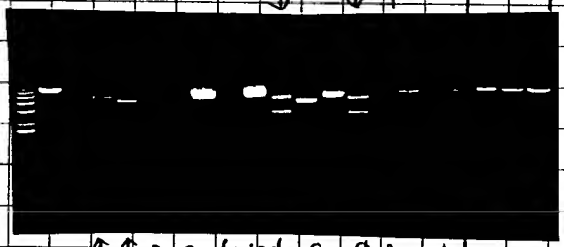
for F8 plasmids (DLZ6)

cut 10 μl plasmid with SmaI and EcoRI at RT / 2 h

EGFP plasmids (DLZ8)

with XbaI + EcoRI

37°C 2 h



—① 1.5 h

I confused C4 & C14, however one of the two has 2 ITRs.

—② 2 h

△ ① DLZ6 colony 10 and colony 13 definitely have two ITRs. I will do more test cuts.

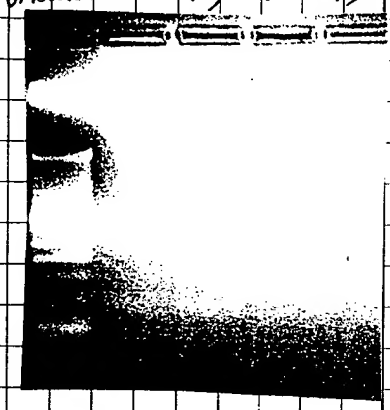
② 6 colonies of DLZ8 seem containing EGFP insert. however this enzyme failed to cut

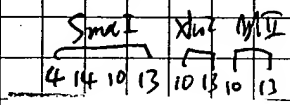
DLZ6

1. Get more plasmids of colony 10 & 13 do more test cuts

① BglII + XhoI & BglII + XbaI

vigna





② SmaI.

~~BglII~~ XhoI.

BglII

C4 has 2 IIRs

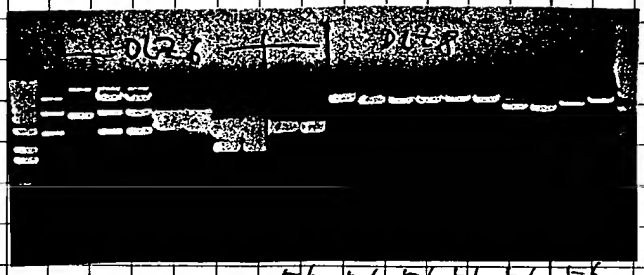
XhoI & BglII cuts of 10 & 13 are ok

Select C13 to sequence.

2. Cut DLZ8. C5 & 6 width more enzyme

the results are confusing.

I will send C6 to ~~for~~ sequence.



① prepare LB media to make more plasmids.

② test-cut xx2.

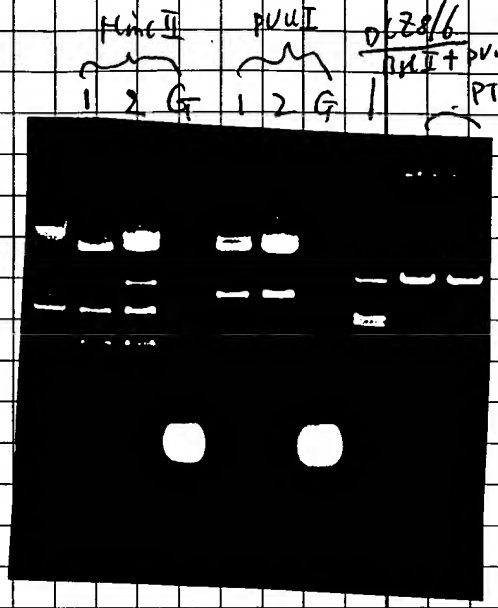
1: colony 1

2: colony 2

G: plasmids xx2 from Genbic.

from HincII & PvuI cuts

the 2 colonies have the same bands with primary plasmids.



③ Transfer EnhancerTRKT MCST EGFPTPA cassette to PTP backbone with two ITRs.

④ prepare culture of plasmids xx2 2 liters

xx6 1 liters

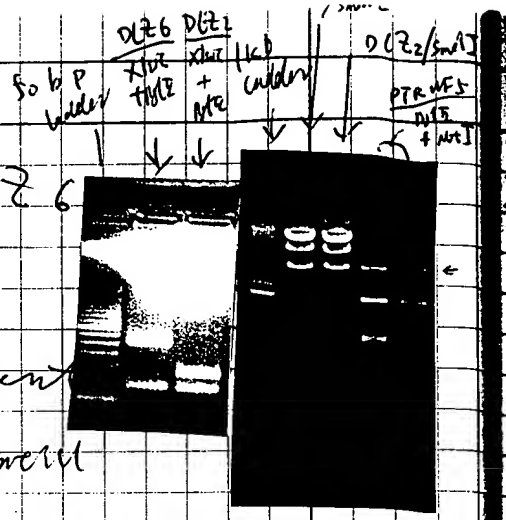
DLZ6-C13 0.5 liters

DLZ2-C3 0.5 liters

⑤ Extract plasmids by Qiagen Mega plasmids kit.

dissolve DNA in TE (pH 8.0)

⑥ plate 96 plates 293 cells (P32)



① test cut of DLZ2 & DLZ6

② 293 cells only 20-30% confluent
perhaps I need to wait overnight
tomorrow for transfection

293 cells : 40-50% confluency

DMEM from Sigma?

Transfection:

① change media at 6:00 pm

② start transfection at 9:00 pm

			12 x
Vector	DLZ2	15 ug	180 ug (450 uL)
	DLZ6	15 ug	(600 uL)
2x2		15 ug	180 ug (150 uL)
2x6		45 ug	540 ug (250 uL)
2.5M CaCl2		125 uL	1500 uL
ddH2O			DLZ2 12.65 mL
			DLZ6 12.50 mL

7.5 mL → 2x HBS

2.5ml per dish. (end at 9:50)
I used vortex machine this time
the precipitate could be seen just 1~2
minutes after mixture.

feed transfected cells with warm
CO_2 – saturated DMEM/10%FBS/Pen.Strep
at 8:30pm

Harvest 293 cells at 12:00 pm.
most of plates reach 90% confluency
(60% confluency at transfect.)
some cells floated.
put the cells to −80°C.

June 11.
Thaw – freeze at 37°–80°C 4 times.
Sonicated.
precipitate the viruses.
Set ultra-spin. 4°C 15°C.

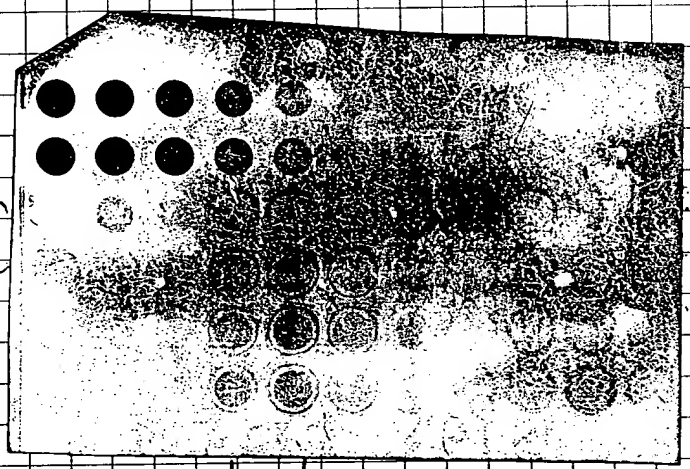
Drip the Gradient of uberva-spin
do doe-blot to titer the virus

Duplicate serum { A, B } 1/100 → ½- serial dilution

Duplicate to wt DE_2 { C, D }

DE_2-6 { E, F }

1. Develop the membrane



Tube 4 5

	desired	
①	100 ng	2.35×10^{10}
②	20 ng	4.2×10^{9}
③	4 ng	9.4×10^{8}
④	0.8 ng	1.88×10^{8}
⑤	0.16	3.76×10^{7}
⑥	0.032	7.52×10^{6}

$\frac{1.88 \times 10^{8}}{\text{vol}} \times 1100\,\mu l = 2.82 \times 10^{10}$

pour 4 & 5 together. dialyze against virus-Dialysis-Buffer overnight

2. plate 2×10^{5}/well & 1×10^{6}/well HepG2 cells (P_{20}) in 6-well plate

Monday

① prepare media for F8 cell culture

a. I screen the F8 in FBS last Friday by APTT

UCRD: 1:40 26.4
1:80 91.4
1:160 103.9
1:320 104.9
1:640 142.4

Serum: Tube 1 2 3
149.9/142.4 135.9/146.K 102.4/108.9

At least I can use serum in tube 1 and 2

b. ~~DEP~~ 10% FBS + Penicillin/streptomycin + 20ug/ml aprotinin (Sigma)

500 ml ~~DMEM~~ Minimal Essential
60 ml FBS
600 ul 100X P/S

100 ml 10% FBS/DMEM + PS → 20ug/ml
2 ml 1mg/ml aprotinin

C infect the HepG2 cells

I will use 1×10^6/well cells

2×10^5 seem a little bit less. and the HepG2 cells grow slower than 293 cells,

There should be 5 days for them to confluency

around 3:00pm

① wash the wells with DMEM

② Add the viruses

DLZ2 : 1 ml → 2

DLZ6 : 1 ml → 5

(1) (2) (3)

(4) (5) (6)

37°C for 2 h swirl plates every 15'

③ suck off ~~medium~~ viruses

add 2ml of MEME to each well

10% FBS, P&G

Aprotinin

△ The ~~construct~~ DLZ8 → EGFP + Neo insert

Worked bad

I could get the expected band by BglII cut

but not from HindIII XbaI. XhoI etc cuts

sequence failed.

I donot know the reason | On the map of BM Constructs, because there is another BamHI site behind polyA of P(Z), so the 3' part of MCS and the entire polyA are deleted. Revin

~~from last Tuesday — Friday~~

I prepared more pTREGFP plasmids.

transform ~~screen~~ the bacteria.

pick colonies (4)

overnight culture — mini — prep.

① the plasmids do not have Bcl I site!

② I cut the EGFP + Neo + poly (A) by XbaI + HpaI

③ sequencing of the DCZ8 — C4 & C8 show the right insert of MCS. I cut C4-8 with XbaI + BamH1.

④ ligation

4 ul ddH₂O
① DCZ8 4 ul
② pTREGFP colony 4 4 ul
③ ① + ②

16°C overnight.

Transform DH5α competent cells this afternoon. 100 ul bacteria / dish

Tuesday

1. transformation of DCZ8

No colony on plate ①

about 20 colonies on plate 2 → contamination from pTREGFP only once cut by XbaI or HpaI?

> 200 on plate 3

pick 8 colonies from plate 3 overnight culture

2 feed HepG2/F8-VMV cells with DMEM+10% FBS PBS + 20 ug/ml Aprotinin some the changed medium at 8pm

3 Count the cells at 3:00 pm.

There are seed 1×10^6 HepG2 cells/per well

48 hours later

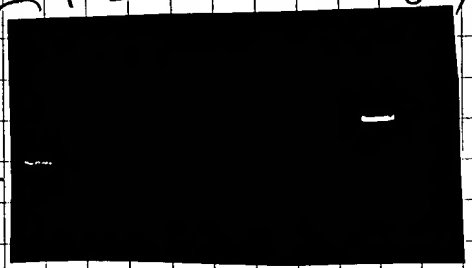
48h. 3:00pm) : 3.2×10^6.

2×10^5 HepG2 cells. 48h later. 3.8×10^5

Excract the plasmids with Promega quiard mini-kit

(cut with XbaI + BglII = 321 + 2493 + 3123)

first I cut the plasmids with XbaI + HindIII and expected a 2.7kb + a 3.3kb bands actually I only could get a 6.0kb bands which mean only XbaI or HindIII site along this plasmid. just as I got before



8 / XbaI + HindIII

I Reviews the maps of pIZx-Cx and pTR-EGFP found there is another BamH1 site between p.polyA and ITR. that means the 3' part of MCS at the poly(A) in pIZx-Cx were deleted. So That is the version.

Recut the plasmids pIZ8 colony 1~8 but 2. with XbaI + HindIII

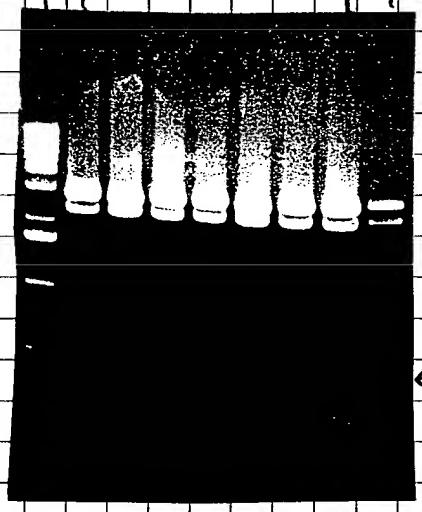
pTR-EGFP / XbaI + HindIII

1kb ladder

pIZ8

pTREGFP

1 th



2-5 th

from pIZ8 a 3200bp band means EcoRI+Tk +5' MCS

pTREGFP a 720bp ——— CMV promoter

Confirm the insert of EGFP to vector pIZx-Cx

① Split of the supernatant of Hep G2/FS

uCRP. 200 ng × 103% FS = 206 ng/ml

1:10	100% activity	
1:20	50%	
1:40	25%	
1:80	12.5%	91.4 seconds
1:160	6.25%	102.4/105.9/102.0/99.4
1:320	3.12%	122.4/118.9/119.4
1:640	1.6%	138.9/138.9/138.9

post-infect

	1×10^6 Hep G2/None	BSU/DLL2 2×10^4/cel	BSU/DLL6 2×10^4/cel
Day 1	142.9	—	132.4/144.9
2	—	—	112.9/123.9
3	144.6/144.9	139.0/142.3	99.4/103.9/102.9
4	143.4/139.4	128.4/132.4	113.9/111.9/103.9/115.9

I can see 3~6% activity of FS in the overlay of cells. remain to be confirmed by elisa & Coacest assays.

transfer Hepic cells to 10-cm-dish in 5ml medium.

② Digest DLZ8 (without 2 DTRs) with

a. BglII to get EnhI + TK + EGFP + Neo + PolyA cassette.

b. XbaI + BglII to confirm EnhI + TK replacement of CMV promoter in PTR-EGFP plasmid

1% agarose gel.

(1)(2) > DLZ8 / AseI + BglII (~~2487+3123~~ 184 + 1235 + 1254 + 2864)

3 PTR-EGFP / BglII + XbaI (615 + 2487 + 3123)

4 DLZ8 / BglII + XbaI (321 + 2493 + 3423)

③ Cut the bands, elute DNA in band purif...

④ Ligate:

① PTR / BglII + CIP 4ul + 4ul ddH2O

② EnhI+TK+EGFP+Neo+Poly / BglII 4ul + 4ul ddH2O.

③ Vector + insert
4ul 4ul.

1ul T4 ligase + 1ul 10x ligase buffer

16°C overnight

Add 10 ml TE (pH 8.0) to the tiger scan use 10 ml to transform DH5α cells. 100 ml/dish Number 4 dish is paul's hepG2 RNA plasmid.

① Transformation:

plate # ① No colony

② < 10 colonies ②

③ > 200 colonies

④ > 500 colonies

pick 8 colonies from plate # 3

4 4

→ 3 ml LB/25 μg/ml Cb 37°C overnight

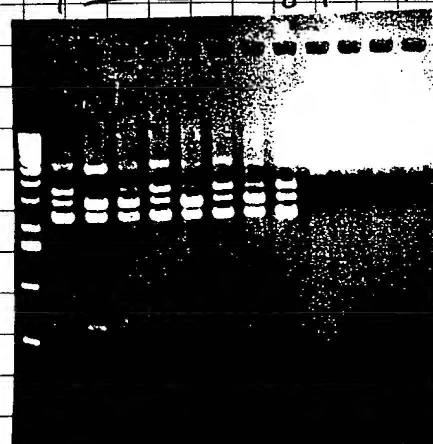
② HepG2 cells/DL26 died. No reasons known. I freeze the cells to see whether I can get some RNA for Northern blot

Extract the plasmids by Promega's miniprep kit

elute DNA in 50 ul ddH2O.

use 10 ul of each to Digest with SmaI

DLZ8 1 — 8 hufIX 1 — 4



1kb ladder

for DLZ8

both ITRs < 638bp, 2264bp, 2945bp

have 5' ITR < 638 bp, 5370 bp

3' ITR < 2264, 3323

So the colony 3, 5, 7 have more intact 2 ITRs

for hufIX plasmids, only one ITR there. I will pick another 8 colonies to screen ITRs.

if there still no colony with 2 ITRs, I need talk with Chris.

use DLZ8 — C5 to make more plasmids

B/25 ug/ml Amp 37°C overnight.

8. pTRUF5 by Qiagen Mega kit

ml TE (pH8.0)

DNA/Oligo Quant

ReadSamples RawData Method SaveClear Print Quit

Results file: A:\WORK_RES Method name: A:\DSDNA

Assay type: ds DNA
Sampling device: None
Read average time: 0.50 sec

Background corr: [Yes] 320.0nm
Pathlength: 1.0000 cm
Conc. factor: 50.000 at 260.0nm

Sample ID	Net Abs 260.0nm	Net Abs 280.0nm	260.0/280.0	Dil. Fact.	Conc. ug/mL
PTRUF5	0.1562	0.0857	1.8231	150.00	1171.4393
DLZ8	0.2561	0.1337	1.9152	150.00	1920.9821

① try to prepare more XX6 and DLZ6 plasmids by CsCl2 gradient methods

However, the yield of plasmids were low.

From 1 liter media only get 0.5 mg plasmids.

BECKMAN DU 640

Time: 11:46

DNA/Oligo Quant
ReadSamples RawData Method SaveClear Print Quit

Results file: A:\WORK_RES Method name: A:\DSDNA

Assay type: ds DNA
Sampling device: None
Read average time: 0.50 sec

Background corr: [Yes] 320.0nm
Pathlength: 1.0000 cm
Conc. factor: 50.000 at 260.0nm

Sample ID	Net Abs 260.0nm	Net Abs 280.0nm	260.0/ 280.0	Dil. Fact.	Conc. ug/mL	
DLZ6	0.0685	0.0359	1.9112	150.00	514.0432	x2ml
XX6	0.0274	0.0124	2.2095	150.00	205.4807	x2ml

② Tried 3 times to plate some Hela cells to test HAV-3 Ab in two dog's plasma, the Hela cells died. no Reasons known. meanwhile the 293 cells died when plated in six-well plates or 12-well plates but alive in 15-cm dish.

medin: Gibco/BRL. DMEM-H, +10% FBS. with or without Ampicillins.

① plate 50 plate of 293 cells (~~grow~~ P30, from vector), cells grow well, but slower than usual. when plated the cells in 6-well plates, died. no reasons known. I have frozen 20 vials of cells in liquid nitrogen. I wonder the viability of them. [illegible]

② Transfection.

30 plates of pCZ8.LEGFP with Eh(TTK)

		1X	30X	
Sol. I	Vector (pCZ8)	15 μg	450 μg	232 μl
	XX2	5 μg	150 μg	116 μl
	XX6	30 μg	900 μg	346 μl
	2.5M CaCl2	0.125 ml		3.75 ml
+	ddH2O			33.051 ml
				37.5 ml

Sol. I 20 plates of pCZ6-but 8.

	20X	
vector	300 μg	584 μl
XX2	100 μg	22 μl
XX6	600 μg	230 μl
2.5M CaCl2		2.5 ml
ddH2O		21.6[illegible] ml

2.5 ml Solu I (1.25 ml for one plate) mix with 2.5 ml 2× HeBS, when the precipitates were seen, add 2.5 ml to each plates

(3) Feed the transfected cells with pre-warmed and CO_2-saturated media/10% FBS on the next day of transfect. ( )

the EGFP cells (green cells) did not appear 8 hours post-transfect.

very significant 20 h post-transfect.

from percentage of green cells in transfected dishes, the transfect efficiency are around 60-70%, similar to the former.

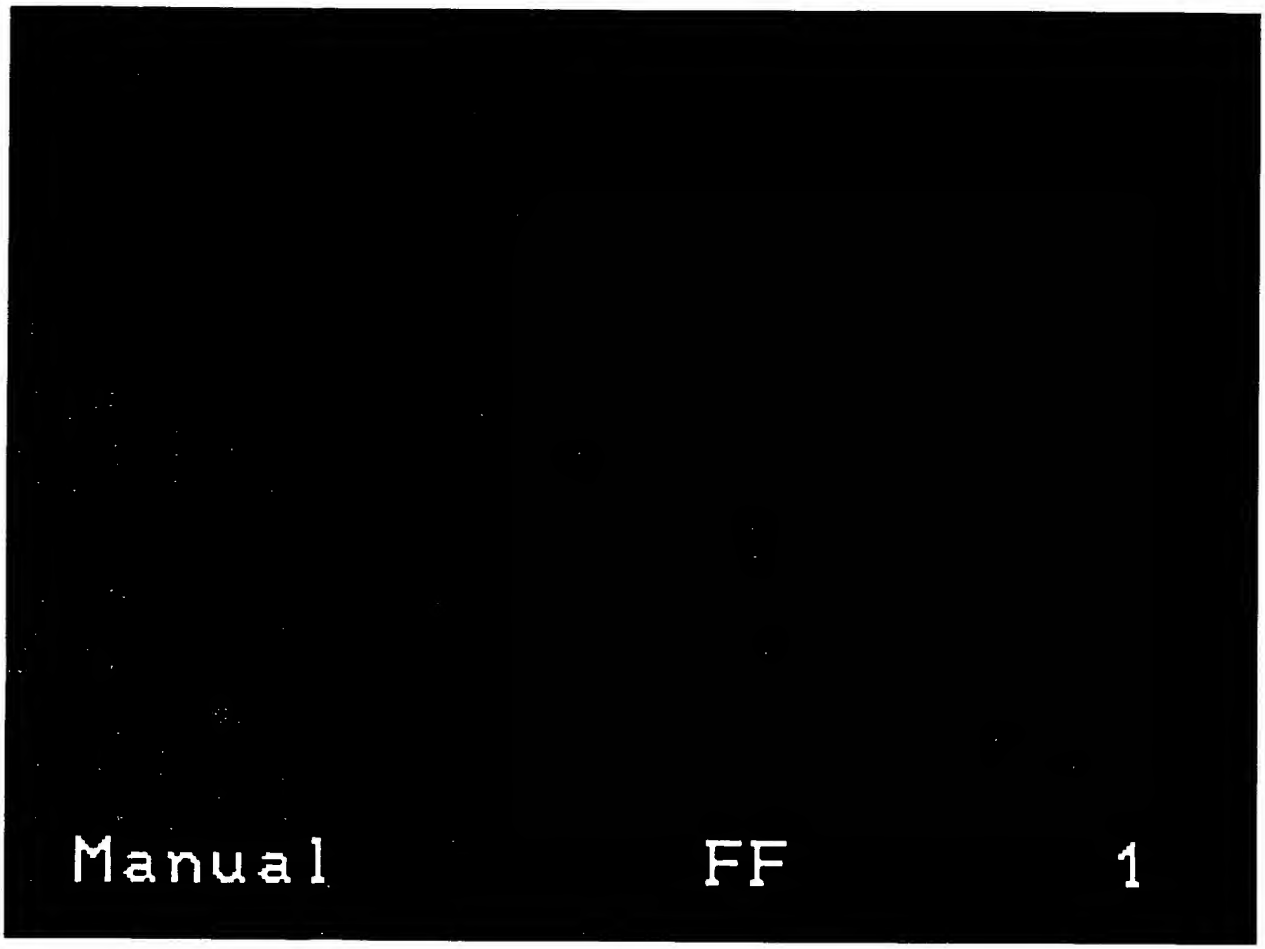
(4) Get Rat liver cell lines

from Dr. Coleman's Lab (Brinkhous-Mulock RLG)

using Ritcher I-10/10% FBS

(Gibco/BRL)

Transfected 293 cells with DCZR



① harvest the making AAV 293 cells release the viruses and set ultra-spin 60 K. 41K. 15°C

② get EGFP template for probe labelling

cut the bands purify them Qiagen gel kit. elute DNA in 100 ul 10mM Tris/(pH 8.0)

EG

← EGFP + Neo

pLZ8/Xba I + BglII

① label the EGFP with Dig following routine protocol

② drip the CsCl2 gradient of rAAV/EGFP & F8.

set dot-blot. 10 ul of each well.

1 2 3 4 5 6 7 8. 9. 10 11. 12

plasmid pLZ8 standard A

AAV EGFP/tube 1 B

2 C

pLZ8 standards D.

plasmids pLZ6 standard { E F

AAV/F8 tube 1 G

tube 2 H

① develop color of dye-blot

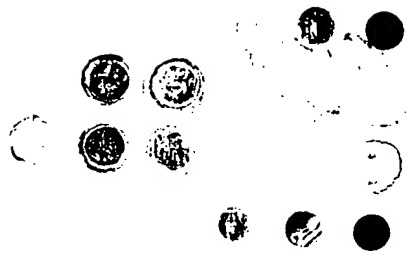
② Dialysis RPMI. < DL26: 2 tube 4.
DL28: 2 tube 5.
change dialysis buffer after 6 hs.

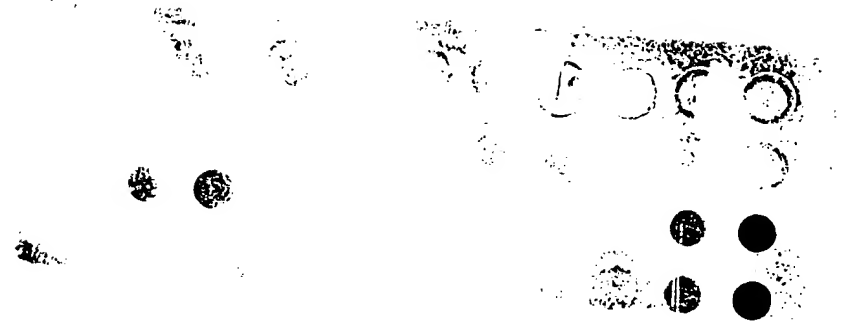
③ plate cells for activity-assay of hu-Ig.

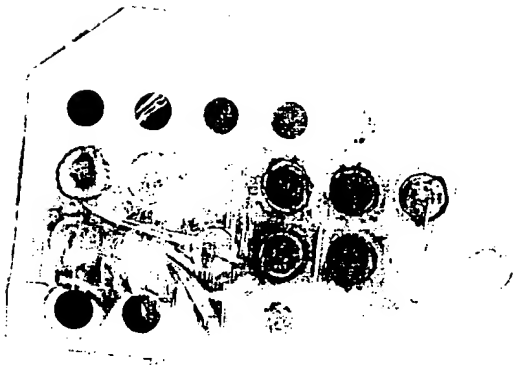
①	②	③
④	⑤	⑥

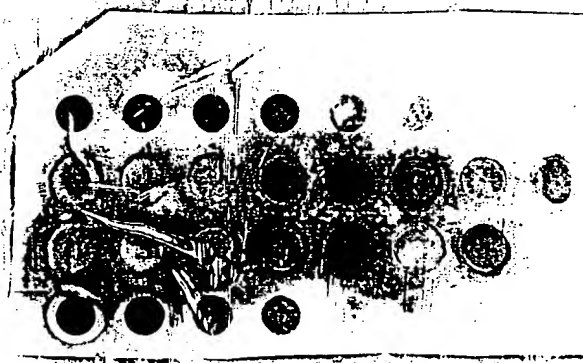
①/④ 2×10^5 293 cell
②/⑤ 4×10^5 HepG2
③/⑥ 4×10^5 WRF344

293 cell : 10% FBS + DMEM.
HepG2 : 10% FBS + MEM α.
WRF344 : 10% FBS + Ham's F-10 (Gibco/BRL)



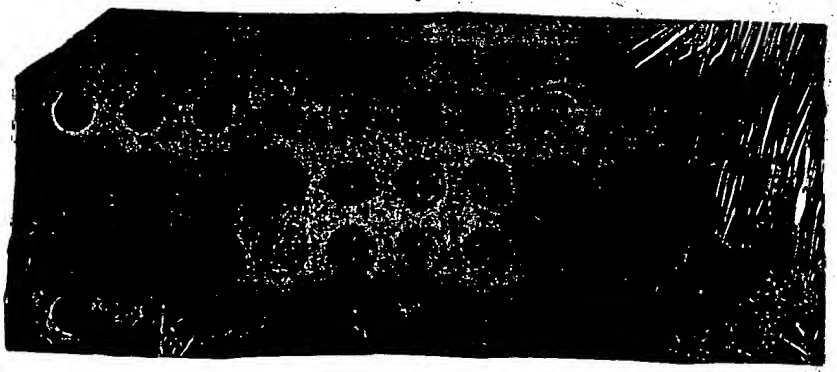






↑ DLZ6

↓ DLZ8



④ functional titer of rAAV/DC28 (GFP)

a. physical titer of rAAV/ps8

$= \frac{2 \times 10^9}{10\,ml} \times 3\,ml = 6 \times 10^{11}$ particles

b. rAAV/GFP $= \frac{3.5 \times 10^9}{10\,ml} \times 6.25\,ml = 2.36 \times 10^{12}$

	DU26	DC28
100 ng	2.35×10^{10}	8.1×10^{10}
20 ng	4.7×10^9	6.2×10^9
4 ng	9.4×10^8	1.24×10^9
0.8 ng	1.88×10^8	2.48×10^8
0.16 ng	3.76×10^7	4.96×10^7

c. 1×10^5/well Hela cells in 12-well plate

12 hours post-plating, Ad MOI = 10 1 h

well ① 10 ml rAAV/GFP + 500 ml DMEM 10 ml

well ② 10 ml ① + ——— 1 ml

well ③ 10 ml ② + ——— 0.1 ml

well ④ 10 ml ③ + ——— 0.01 ml

20-30 green cells in each field

1-2 green cells in some field

No. green cells

infect cells with HBV/HepG2 (OLYB)

the viruses I used is from tube 4.

the titer is $\frac{2 \times 10^7}{10\,ml} \doteq 2 \times 10^6$/ml.

so. for the 293 cells (2×10^5) : 100ml

+900ml DMEM

HepG2-WRF344) (4×10^5) 200 ml

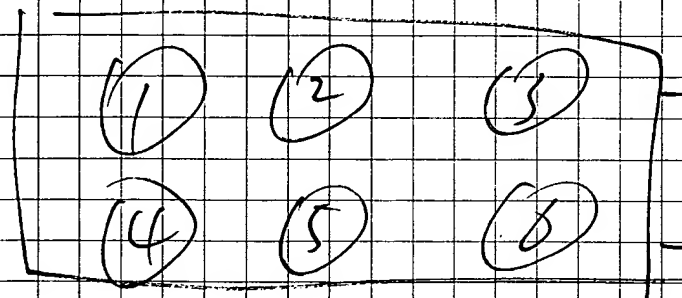
+800ml DMEM.

① wash the ~~plate~~ each well with 2ml DMEM.

② Add the viruses, incubate at 37°C for 1 h. rock the plate every 15 m.

③ suck off the viruses, wash the well with DMEM.

④ add 2ml relevant media/PBS to each well. (at 12:00 finish infect)



Remark: ① 293 cells, most were detached, and some more lost during the course.

② 4×10^5/well WRF344 are too heavy confluency, they are nearly 100% confluency while infection.